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INHIBITION OF GAMETE MEMBRANE FUSION IN THE SEA URCHIN BY QUERCETIN

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ABSTRACT

We have treated sea urchin gametes with quercetin to investigate the effects of this drug on fertilization and egg activation. High concentrations of this drug gradually inhibited sperm motility, but pretreatment of sperm with the drug did not affect their fertility. Treatment of eggs with quercetin at micromolar concentrations completely blocked fertilization. The drug did not block the acrosome reaction of sperm, nor did it affect the binding of the acrosomal filament to the vitelline layer of the egg. However, fertilization was prevented. Inseminated quercetin-treated eggs could be activated by ionophore A23187, but still failed to incorporate sperm. Quercetin blocked fertilization if added after primary gamete binding, but 30 s before the beginning of the cortical reaction in experiments in which eggs were inseminated at low temperature. Drug added 10 s before the beginning of the cortical reaction was ineffective. Electron microscopy confirmed normal primary gamete binding and failure of fertilization. Vitelline layer removal by trypsin did not restore fertilizability to quercetin-treated eggs. Thus quercetin prevented fertilization by blocking gamete membrane fusion. These results indicate that quercetin can be a useful probe for the mechanism of membrane fusion in fertilization.

INTRODUCTION

There are two generally recognized steps in sperm-egg attachment in the sea urchin; primary gamete binding and membrane fusion. The first is the attachment of the surface of the reacted acrosome to the vitelline layer (Summers *et al.*, 1975). This interaction results from a previous interaction between a fucose-sulfate polysaccharide of the egg jelly coat (SeGall and Lennarz, 1979) and a glycoprotein of the sperm surface (Saling *et al.*, 1982; Eckberg and Metz, 1982). After the acrosome reaction, bindin on the acrosomal filament of the sperm (Vacquier and Moy, 1977) and a glycopeptide receptor of the vitelline layer (Glabe and Vacquier, 1978) cement the gametes together.

The binding of the fertilizing sperm to the egg surface results in a rapid depolarization of the egg surface (Jaffe, 1976; Chambers and deArmendi, 1979) and a rapid block to polyspermy (Jaffe, 1976; Schuel and Schuel, 1981). Subsequent intracellular calcium release leads to the cortical reaction and the activation of egg metabolism (Epel, 1982). Sperm incorporation into the egg is due to the action of microfilaments in egg microvilli. These form the fertilization cone which surrounds the sperm and actively engulfs it (Longo, 1978; Schatten and Schatten, 1980).

We have recently shown that quercetin, an ATPase inhibitor, can initiate meiosis in *Chaetopterus* oocytes (Eckberg and Carroll, 1982). Since meiosis initiation is

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Abbreviation: ASW, MBL formula artificial sea water.

believed to be dependent upon a transient calcium flux within the oocyte (Moreau *et al.*, 1978), we concluded that quercetin might act to initiate meiosis by increasing the intracellular free calcium concentration as the result of inhibition of calcium sequestration. Since such an increase activates sea urchin eggs, we wished to determine whether quercetin might activate sea urchin eggs even though the drug was not found to be parthenogenetic in *Chaetopterus*. Quercetin did not activate sea urchin eggs. Instead, the drug blocked fertilization by sperm, but not activation by ionophore A23187. The results indicate that the drug acts to prevent gamete membrane fusion. A preliminary account of this work has appeared in abstract form (Eckberg and Perotti, 1982).

Materials and Methods

Gametes of the sea urchins *Arbacia punctulata* (Marine Resources Division, Marine Biological Laboratory, Woods Hole, MA), *Lytechinus pictus* and *Strongylocentrotus purpuratus* (Pacific BioMarine Laboratories, Venice, CA) were used in these experiments. Most of the experiments were performed on at least two of the species with quantitatively similar results. *Arbacia* gametes were obtained by electrical stimulation; *Lytechinus* and *Strongylocentrotus* gametes were obtained by KCl (0.5 *M*) injection. Experiments on *Arbacia* and *Lytechinus* were performed at 22–23°C; those on *Strongylocentrotus* were performed at 14–18°C. MBL formula artificial sea water adjusted to pH 8.2 with 10 m*M* Tris-HCl (ASW) was used throughout. Egg vitelline layers were disrupted by trypsin digestion (Saling *et al.*, 1982).

Stock solutions of quercetin (10 m*M*) and ionophore A23187 (1 mg/ml) in ethanol were added to eggs as described (Eckberg and Carroll, 1982). Sperm were added after incubation of eggs in quercetin for 5 min, except where noted. Ethanol had no effect on the phenomena observed at the concentrations tested. To assay for the loss of sperm motility in quercetin, concentrated semen was suspended in ASW to 0.1% (v/v). Various concentrations of quercetin were added and the sperm suspensions were examined at intervals for motility as described (Saling *et al.*, 1982).

To determine sperm binding to eggs in quercetin, eggs were fixed (Eckberg, 1981) at intervals after insemination or A23187 activation and examined by phase-contrast microscopy. For electron microscopy, eggs were postfixed and embedded (Eckberg, 1981), sectioned with a Reichert OM3 ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Siemens Elmiskop 101 electron microscope.

To assay for sperm incorporation, inseminated eggs were fixed in ethanol/glacial acetic acid (3:1, 3 changes), cleared with 45% acetic acid, stained with aceto-orcein and examined microscopically for incorporated sperm. Since sperm attached to the surface of unfertilized eggs in quercetin interfered with such counts, eggs were treated with ionophore A23187 prior to fixation to initiate the cortical reaction and thus to remove attached but unincorporated sperm. Embedded and sectioned eggs were also examined for incorporated sperm by light and electron microscopy.

Results

Figure 1 shows that micromolar concentrations of quercetin effectively blocked fertilization. Concentrations at or above 30 μM were completely effective, while those at or below 3 μM had no effect.

Although quercetin was routinely added to egg suspensions 5 min prior to insemination, Figure 2 shows that the drug exerted its effects on fertilization very

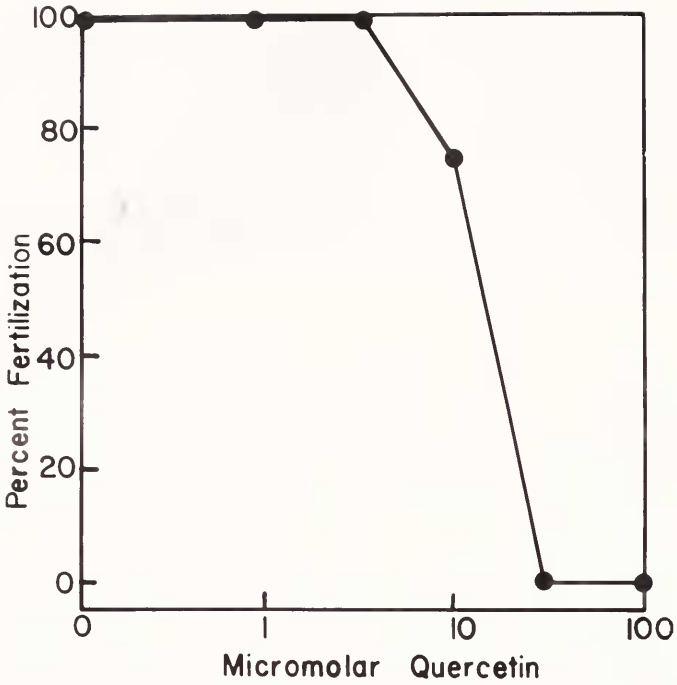


FIGURE 1. Effect of quercetin on fertilization of *Lytechinus pictus* eggs. Eggs were mixed with various concentrations of quercetin in ASW and inseminated five min later. Eggs were examined by phase-contrast microscopy and scored as fertilized if they showed elevated fertilization envelopes, post-fertilization nuclear changes and cleavage.

quickly. Quercetin significantly inhibited fertilization in *Strongylocentrotus* at 14°C when added as late as 20 s after insemination, whereas additions 40 s after insemination were ineffective. Eggs which became fertilized in quercetin subsequently cleaved, indicating that the drug did not directly affect either sperm incorporation, pronuclear migration and fusion, or cell division.

Quercetin treatment was reversible. Sperm or eggs pretreated with 100 μM quercetin and washed with ASW were fertile. Furthermore, inseminated eggs in quercetin could become activated if the quercetin was subsequently diluted with ASW.

Figure 3 shows that quercetin inhibited sperm motility. At fertilization-inhibiting concentrations, sperm motility was reduced within 3 min and ceased with an I_{50} (time of 50% inhibition) of *ca.* 10 min. Lower concentrations of quercetin had no significant effect on sperm motility.

Light and electron microscope analyses showed that sperm could undergo the acrosome reaction in quercetin and bind to and probably penetrate egg vitelline layers but could not activate eggs. Control *Lytechinus* eggs fixed 30 s after insemination had begun to undergo the cortical reaction, but still had numerous sperm bound (Fig. 4). Quercetin-treated eggs fixed at the same time after insemination showed bound sperm but no evidence of egg activation (Fig. 5). Electron microscopy confirmed the fact that egg activation had not begun since all cortical granules were intact (Fig. 6). Electron microscopy also showed that sperm bound to the eggs were attached by the tips of their acrosomal filaments (Fig. 7), and that quercetin had interfered with neither the acrosome reaction nor vitelline layer attachment (primary gamete binding).

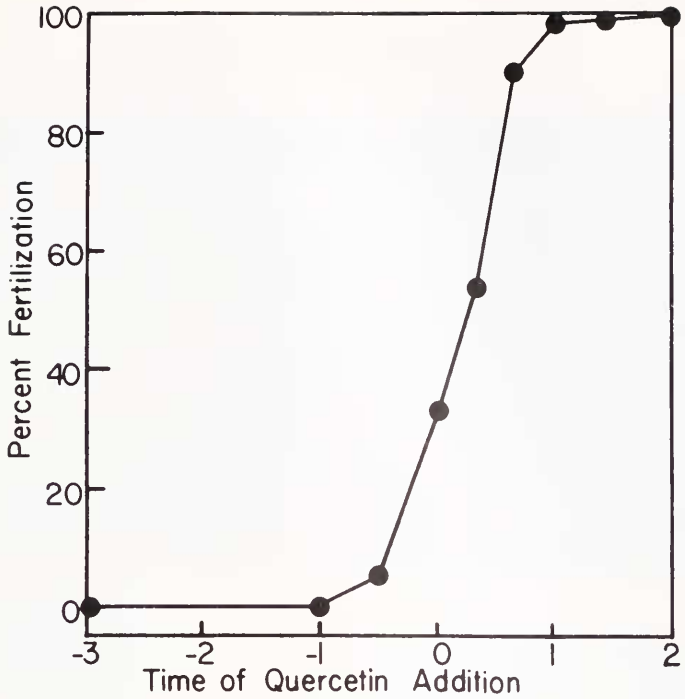


FIGURE 2. Effect on time of quercetin addition relative to time of insemination on egg activation in *Strongylocentrotus purpuratus*. Sperm were added at "0" and quercetin (100 μM) was added at the times indicated relative to sperm addition. Times are given in minutes. Fertilization was determined microscopically as described in the legend to Figure 1.

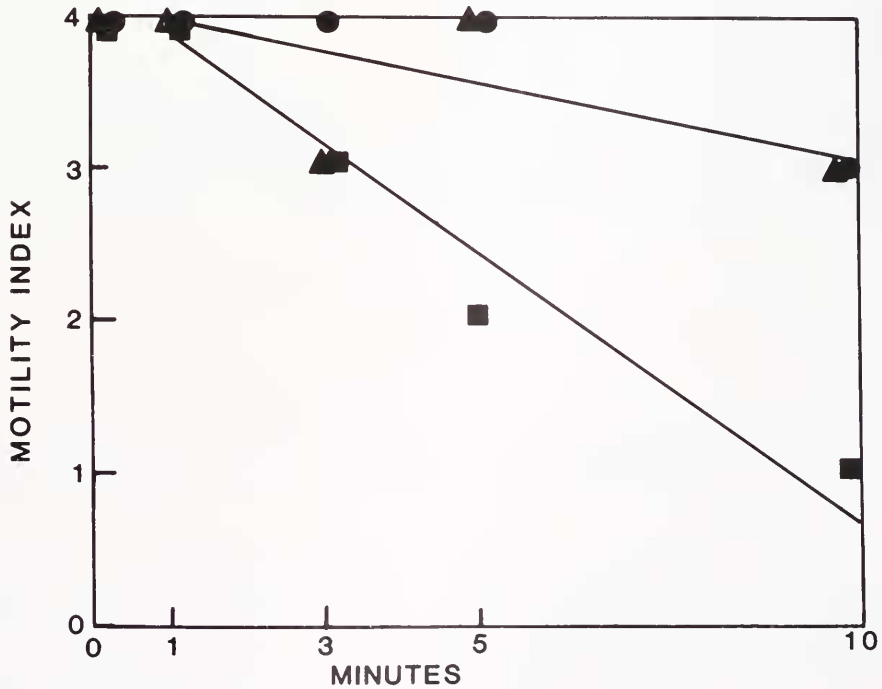
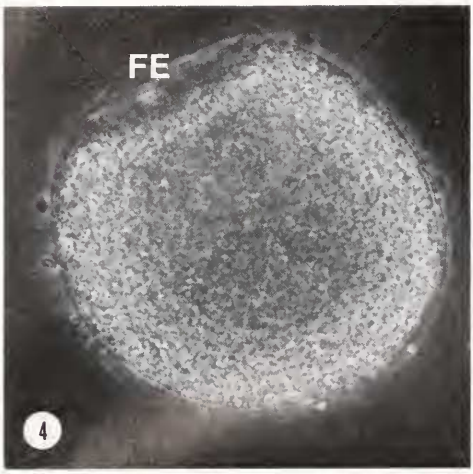
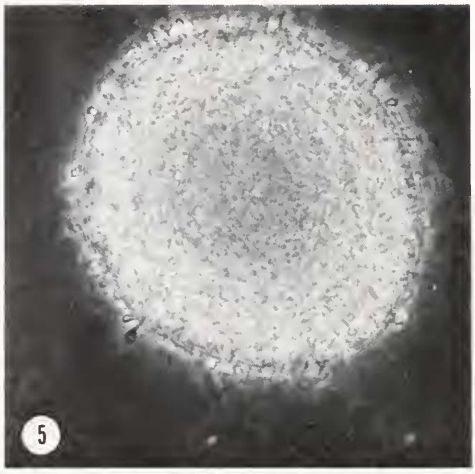
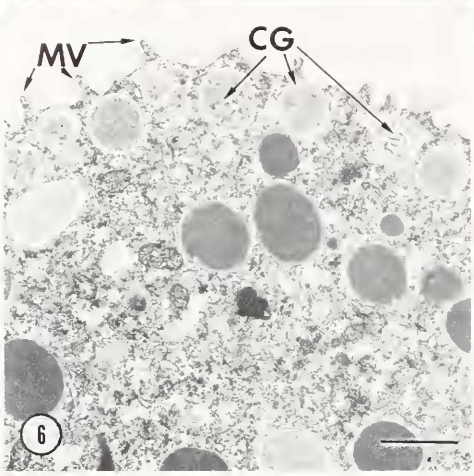


FIGURE 3. Effect of quercetin on the decay of motility by *Lytechinus pictus* sperm. Sperm were incubated either in the absence of quercetin (circles) or in the presence of 10 μM (triangles) or 100 μM (squares) quercetin. Sperm were examined at intervals for motility by phase-contrast microscopy.







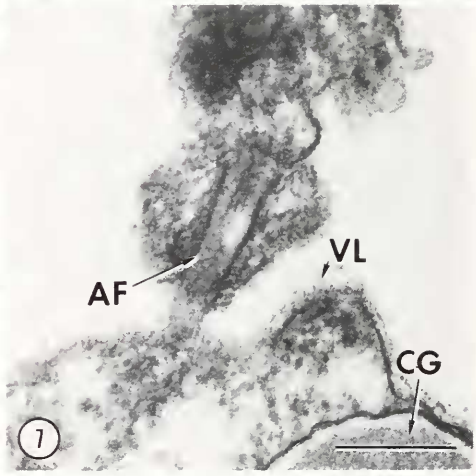


FIGURE 4. *Lytechinus pictus* egg 30 s after insemination in ASW. Note the beginning of fertilization envelope (FE) elevation. Also note that sperm are attached wherever the fertilization envelope has not yet begun to elevate.

FIGURE 5. *Lytechinus pictus* egg 30 s after insemination in 30 μM quercetin. Many sperm are attached to the vitelline layer of the egg, but there is no evidence of egg activation.

FIGURE 6. Surface of a quercetin-treated *Lytechinus pictus* egg. The surface shows typical microvilli (MV) and the cortical granules (CG) are intact. Bar = 1.0 μm.

FIGURE 7. Electron micrograph of a *Lytechinus pictus* sperm bound to the surface of an egg 30 s after insemination in 30 μM quercetin. The acrosomal filament (AF) has attached to the vitelline layer (VL) but the acrosomal process is still surrounded by a continuous membrane. An intact cortical granule (CG) can be seen adjacent to the attached sperm. Bar = 0.2 μm.

If vitelline layer penetration were prevented by quercetin, disruption of the vitelline layer should restore fertilizability. However, trypsin disruption of the vitelline layer did not restore fertilizability (Table I).

By 5 min after insemination, control eggs showed normal fertilization envelopes (Fig. 8), where quercetin-treated eggs still showed attached sperm but no evidence of activation (Figs. 9, 10). Subsequent treatment with ionophore A23187 resulted in fertilization envelope elevation and sperm detachment (Fig. 11). Electron mi-

TABLE I

Effect of quercetin on fertilization of vitelline layerless eggs of Arbacia punctulata.

Vitelline layer	Quercetin	Percent fertilized
present	none	100
present	100 μM	0
absent	none	97
absent	100 μM	0

croscopy confirmed that all cortical granules in these eggs had discharged (Fig. 12). Such eggs were examined for evidence of sperm penetration by light microscopy of cleared whole mounts and sectioned embedded material and by electron microscopy. In no case did we observe any evidence of sperm penetration into the eggs.

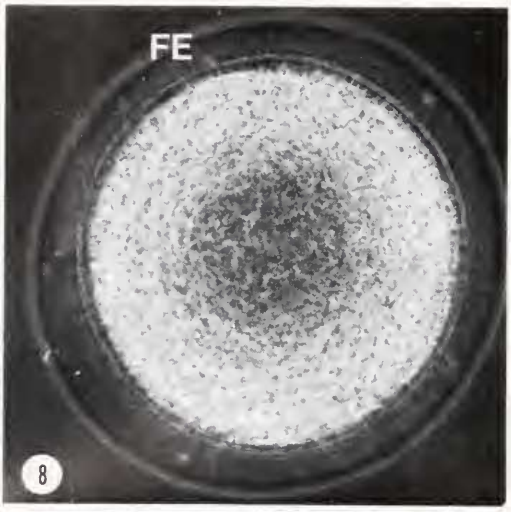
DISCUSSION

Treatment of eggs with 30–100 μM quercetin blocked fertilization without significantly affecting sperm-egg binding. Although quercetin inhibited sperm motility, this was evidently not the reason for the inhibition of fertilization. Sperm motility decayed only after 1–2 min, after the time at which the fertilizing sperm would have activated the egg. Furthermore, preincubation of either sperm or eggs with quercetin followed by dilution or washing did not affect fertilization. Finally, sperm attached to eggs in quercetin, and such attachment was morphologically normal by both light and electron microscopy. Removal or disruption of the vitelline layer did not restore fertilizability to quercetin-treated eggs. Nor was the failure of egg activation due to direct blockage of the cortical reaction, since ionophore-activated eggs could undergo a normal cortical reaction even after prolonged treatment with at least 200 μM quercetin. The lack of a direct effect on the cortical reaction was surprising since quercetin is known to inhibit secretion in other cells (Fewtrell and Gomperts, 1977). This may indicate that cortical granule exocytosis is partially controlled by a different mechanism than secretion in other cells.

Finally, quercetin did not directly block sperm incorporation. Sperm incorporation is dependent upon microfilaments present in the fertilization cone which forms subsequent to egg activation (Longo, 1978; Schatten and Schatten, 1980). Eggs treated with quercetin after membrane fusion were fertilized, formed asters and pronuclei, and cleaved normally. Quercetin, therefore, had no direct effect on the sperm incorporation apparatus.

Inhibition of fertilization without effects on events up to primary gamete binding and vitelline layer penetration or subsequent to egg activation suggests that quercetin must have blocked some step in the interval between these events. The kinetics of quercetin inhibition of fertilization further indicated that the drug blocked a step at this time. Addition of quercetin to *Strongylocentrotus* eggs up to 30 s before the cortical reaction of control eggs resulted in significant inhibition of fertilization. However, addition of the drug 10-15 s before the cortical reaction was relatively ineffective. Since membrane fusion occurs in this interval and is the only known sperm-egg interaction which cannot be excluded as the affected step, this step must have been blocked.

The mechanism of action of quercetin in inhibiting gamete membrane fusion is not yet clear. Nor is the mechanism of gamete membrane fusion itself known. However, quercetin is known to inhibit membrane enzymes (Fewtrell and Gomperts,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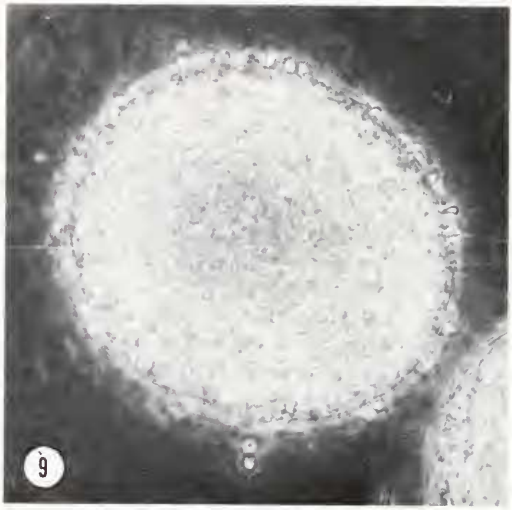
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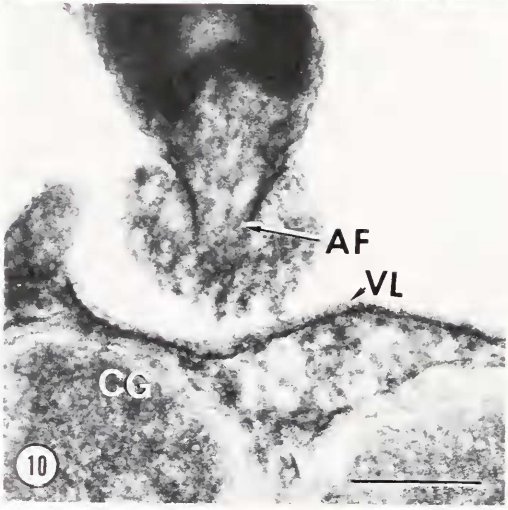
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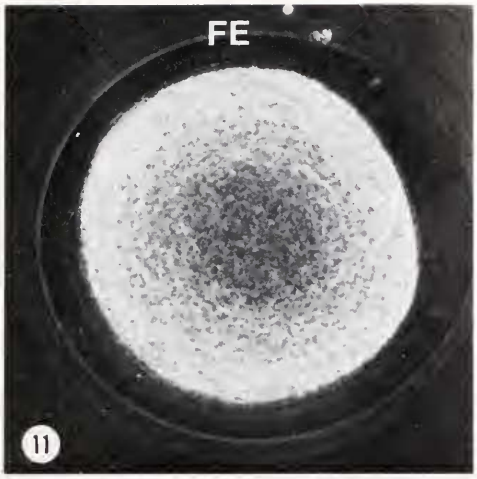

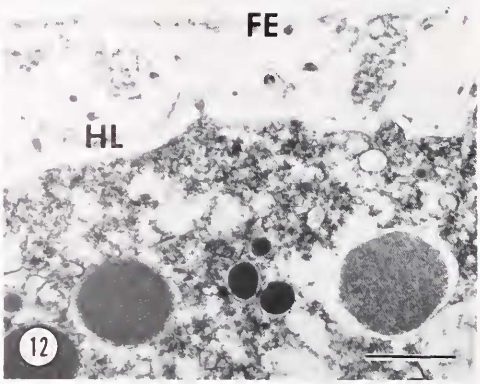

FIGURE 11. *Lytechinus pictus* egg which was exposed to 4μg/ml ionophore A23187. The egg had been treated with 30 μ*M* quercetin for 5 min, inseminated in quercetin and treated with ionophore 5 min later. A fertilization envelope (FE) has formed and sperm have detached.

FIGURE 12. Surface of an ionophore-activated, quercetin-treated egg of *Lytechinus pictus*. Note the presence of a fertilization envelope (FE) and forming hyaline layer (HL) and the absence of cortical granules. Bar = 1.0 μm.

1977), and thus it seems likely that the drug acts by altering the activity or organization of membrane proteins.

The effects of quercetin on gamete fusion may be general. We have recently found that quercetin blocks fertilization in *Spisula* (Eckberg, 1982) and *Chaetopterus* (unpublished data of W. R. Eckberg, L. D. Brown and R. Pettaway). Quercetin probably acts by inhibition of membrane fusion in these species as well.

Fluorescein dyes have recently been suggested to inhibit membrane fusion in sea urchins (Carroll and Levitan, 1978a; Finkel *et al.*, 1981) and in other forms as well (Carroll and Levitan, 1978b). Effectiveness of these anionic dyes was directly proportional to their octanol-water partition coefficients.

The mechanism of action of such dyes that inhibits membrane fusion is unknown, and there is some indication that their action is different from that of quercetin. Erythrosin B but not quercetin decreased primary gamete binding and inhibited egg activation by ionophore A23187 (Carroll and Levitan, 1978a). However, both drugs blocked fertilization when added as late as 20 s after insemination, and both were still effective when egg vitelline layers were disrupted, so they probably both affected gamete membrane fusion. Since quercetin did not affect primary gamete binding or ionophore activation of eggs, quercetin would seem to be a superior drug for inhibition of membrane fusion.

FIGURE 8. *Lytechinus pictus* egg 5 min after insemination in ASW. The fertilization envelope (FE) has completely formed and supernumerary sperm have detached.

FIGURE 9. *Lytechinus pictus* egg 5 min after insemination in 30 μ*M* quercetin. Sperm are still attached and there is no evidence of egg activation.

FIGURE 10. Electron micrograph of a *Lytechinus pictus* sperm bound to the surface of an egg 5 min after insemination in 30 μ*M* quercetin. There has been no change in the mode of sperm-egg attachment. The sperm is still attached by its acrosomal filament (AF) to the vitelline Layer (VL) and the cortical granules (CG) remain intact. Bar = 0.2 μm.

The results of this study indicate that quercetin can be a useful probe for the subcellular components and biochemical processes involved in gamete membrane fusion and fertilization.

ACKNOWLEDGMENTS

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